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Time-kill kinetics of *Piper betle* L. ethanolic leaf extract on methicillin-sensitive *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is one of the main pathogens in community and hospital infections that could cause mild skin infections to severe life-threatening bacteremia. *Piper betle* has shown antibacterial activities against *S. aureus* but its pharmacodynamics remains unclear despite its widespread availability in many over-the-counter preparations. This study aimed to describe the time-kill kinetics of *P. betle* extract against methicillin-sensitive *Staphylococcus aureus* ATCC 29213 (MSSA). Time-kill kinetics were conducted to describe the killing rate of *P. betle* extract by its effects on MSSA lag time duration, growth rate, and maximum optical density. The surviving colonies at different time-points after exposure to *P. betle* extract at minimum bactericidal concentration were also determined. Subinhibitory concentrations elucidated its concentration-dependent antibacterial activity by maintaining a trend of increasing lag time, decreasing growth rate, and decreasing maximum optical density as the concentration increases. In particular, concentration at 1250 µg/mL or 0.5 x minimum inhibitory concentration (MIC) showed consistent significant findings across these parameters. Concentrations at MIC and above did not show growth, indicating MSSA growth inhibition or death. *P. betle* extract's bactericidal effect started immediately within two (2) hours and was sustained until no growth was observed from the eighth hour and beyond. *P. betle* extract maintained antibacterial activities against MSSA at subinhibitory concentrations and has also exhibited immediate and sustained bactericidal effect at minimum bactericidal concentration (MBC).

Keywords: *Piper betle; Staphylococcus aureus; anti-bacterial agents; pharmacologic actions and time-kill kinetics*

INTRODUCTION

Staphylococcus aureus is one of the main pathogens in community and hospital infections that can cause mild skin infections to severe life-threatening bacteremia (Taylor & Unakal, 2021). Invasive methicillin-sensitive *Staphylococcus aureus* ATCC (MSSA) infections have caused significant morbidity and mortality with incidence and associated death exceeding that of methicillin-resistant *S. aureus* (Jackson et al., 2019). Treatment strategies against *S. aureus* remain widely available but research for new antimicrobial agents should continue.

The leaves of *Piper Betle* (*P. betle*) has been used in common ailments by many Asian countries, where *P. betle* is widely cultivated particularly in India, Malaysia, Sri Lanka, and Thailand (Datta et al., 2011). In some communities in India, claims of the superiority of *P. betle* over modern medicine are made, and interestingly, patients from these communities have a reduced risk of acquiring antibiotic-resistant infectious diseases compared to those from urban areas (Datta et al., 2011).

Locally available *P. betle* plant has shown antibacterial property using its various extracts against MSSA and methicillin-resistant *S. aureus* (Balinado & Chan, 2019; Jamelarin & Balinado, 2019; Valle et al., 2015; Valle et al., 2016; Valle et al., 2016). A topical preparation of the *P. betle* leaf ethanolic extract has already been studied and shown activity against *S. aureus*, supporting the possibility of an effective topical drug delivery system in treating or preventing bacterial infections (Budiman et al., 2018; Trakranrungsie et al., 2006; Valle et al., 2021). To date, no time-kill kinetics study was conducted on *P. betle* ethanolic leaf extract that measured parameters such as lag time duration, growth rate, and maximum optical density, which aggravates the lack of understanding of its pharmacodynamics. With this, the study aimed to describe the time-kill kinetics of *P. betle* L. ethanolic leaf extract (PBE) against MSSA that measured parameters including those previously enumerated.

METHODOLOGY

Equipment, reagents and bacterial inoculum

Standard laboratory equipment such as UV-visible spectrophotometer (Thermo Scientific™ GENESYS™ 180) for inoculum standardization and biofilm quantification, rotary evaporator (DLAB RE100-Pro) to concentrate the plant extract, and incubator (Lab Companion) for *S. aureus* cultures were used. A blender (Imarflex) was used to homogenize the dried *P. betle* leaves.

Analytical grade of absolute ethanol (Scharlau) and Mueller Hinton broth (MHB) and trypticase soy agar (TSA) from Hi-Media were used as growth media. Tween 80 and USP-grade oxacillin powder (Sigma-Aldrich) were also procured from a local supplier. The *Staphylococcus aureus* ATCC 29213 was obtained from the Department of Medical Microbiology, College of Public Health, University of the Philippines, Manila.

Extract preparation

The fresh leaves of *P. betle* were collected from the mountaintops located in Bulacan, Philippines by a local supplier. Pesticides were not used during cultivation and the farm was located far from highways and industrial areas. Collection was done in July and November 2021. The *P. betle* plant was authenticated by the Institute of Biology, College of Science in the University of the Philippines Diliman.

The preparation of PBE was based on previously reported method with minor modifications (Valle et al., 2016). *P. betle* leaves were air dried and pulverized into powder prior to ethanolic extraction. A total of 1500 g of powdered *P. betle* leaf was soaked in 6 L of absolute ethanol for 7 days with occasional shaking. After the maceration, the mixture was filtered through a Whatman filter paper No. 1. All the filtrates were concentrated using a rotary evaporator at 50°C at 600 mmHg while rotating at 90 rpm. The extract was then further concentrated under the same temperature using a water bath. This gave a 2.67% extract yield from the dried leaves. The crude extract was stored in a container at -4°C until further use.

S. aureus stock and inoculum preparation

The following procedures were based on standard methods (CLSI, 2012; Missiakas & Schneewind, 2013). Briefly, using a sterile inoculating loop, a small amount of MSSA from the stock was streaked onto a small section of a TSA plate using aseptic technique. A new sterile inoculating loop was passed through the initially streaked quadrant, repeating the process 1-2 more times. The plates were incubated for 16 hours at 37°C.

An overnight growth method was used to prepare necessary broth suspensions containing 1 x 10^8 CFU/mL for the procedures in the in vitro assays. The initial overnight broth culture was standardized at optical density at 600 nm (OD600) to CFU/mL. The computed conversion factor of the OD600 to CFU/mL was the basis of the dilution of the overnight cultures (Wiegand et al., 2008).

For the time-kill kinetics, the initial 1 x 10^8 CFU/mL overnight bacterial culture was diluted to 1:10 in MHB twice to make final concentration of approximately 1 x 10^6 CFU/mL.

Time-kill kinetics

Following standard methods with minor modifications, time-kill kinetics was conducted through the principles of broth microdilution assay and UV-visible spectrophotometer (CLSI, 2012; NCCLS, 1999; Valle et al., 2016; Wiegand et al., 2008). PBE concentrations used in the time-kill kinetics study include the predetermined minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) at 2500 µg/mL and 5000 µg/mL, respectively (Lao et al., 2022).

Two-fold serial dilution of PBE was used at a starting concentration of 200 mg/mL in 10% Tween 80 then using MHB as diluent resulting in final concentrations of 9.8 µg/mL to 10000 µg/mL. Each well of a 96-microwell plate contained 100 µL of PBE and bacterial suspension with approximately 5 x 10^5 CFU/mL of MSSA. This was incubated at 37°C for 24 hours with constant shaking in the microplate reader for determination of OD600 every 15 minutes. A growth control, Tween 80 as negative control, and 2 µg/mL of oxacillin as a positive control were used. A group containing different PBE concentrations and MHB only were used as the blank control. The results was processed by GrowthRates version 6.2 to compute for lag time, growth rate, and maximum optical density (Hall et al., 2014).

Manual time-kill kinetics at MBC

A duplicate 10-mL broth was prepared containing the plant extract at MBC, MHB, and inoculum at approximately 5 x 10^5 CFU/mL MSSA. At 0, 2, 4, 8, 10, 12, and 24th hour, 0.5mL of the broth was serially diluted in four 1:10 dilutions followed by plating 100µL of the last 3 dilutions onto TSA. Colonies between 20-200 were counted and used to compute for CFU/mL (NCCLS, 1999). This was plotted against time to illustrate its bactericidal effect.

Statistical analysis

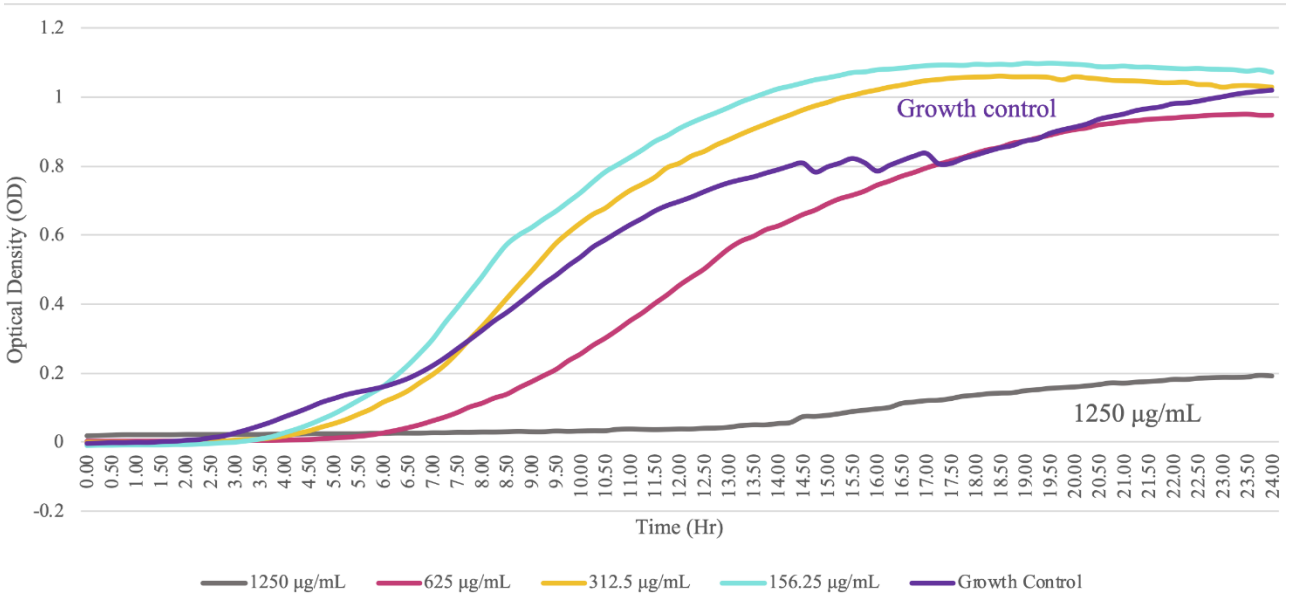
The results were expressed as means for each group and were evaluated using a one-factor analysis of variance (ANOVA), followed by post-hoc analysis using Tukey's HSD. Results were considered to be statistically significant at $p<0.05$. The researcher utilized IBM® SPSS® Statistics version 23 for the statistical analysis.

RESULTS

Subinhibitory concentration of 1250 µg/mL PBE consistently resulted to significant effects on the lag time, growth rate, and maximum optical density of MSSA ($p<0.05$). No growth was noted for oxacillin, blank controls, and in concentrations at MIC and above. Figure 1 shows growth curves of *S. aureus* at different subinhibitory concentrations of the extracts (i.e., 156.25 µg/mL, 312.5 µg/mL, 625 µg/mL, and 1250 µg/mL). Concentrations below 156.25 µg/mL failed to show inhibitory effects on MSSA.

Figure 1

Staphylococcus aureus ATCC 29213 (MSSA) optical densities in different subinhibitory concentrations of Piper betle ethanolic leaf extract

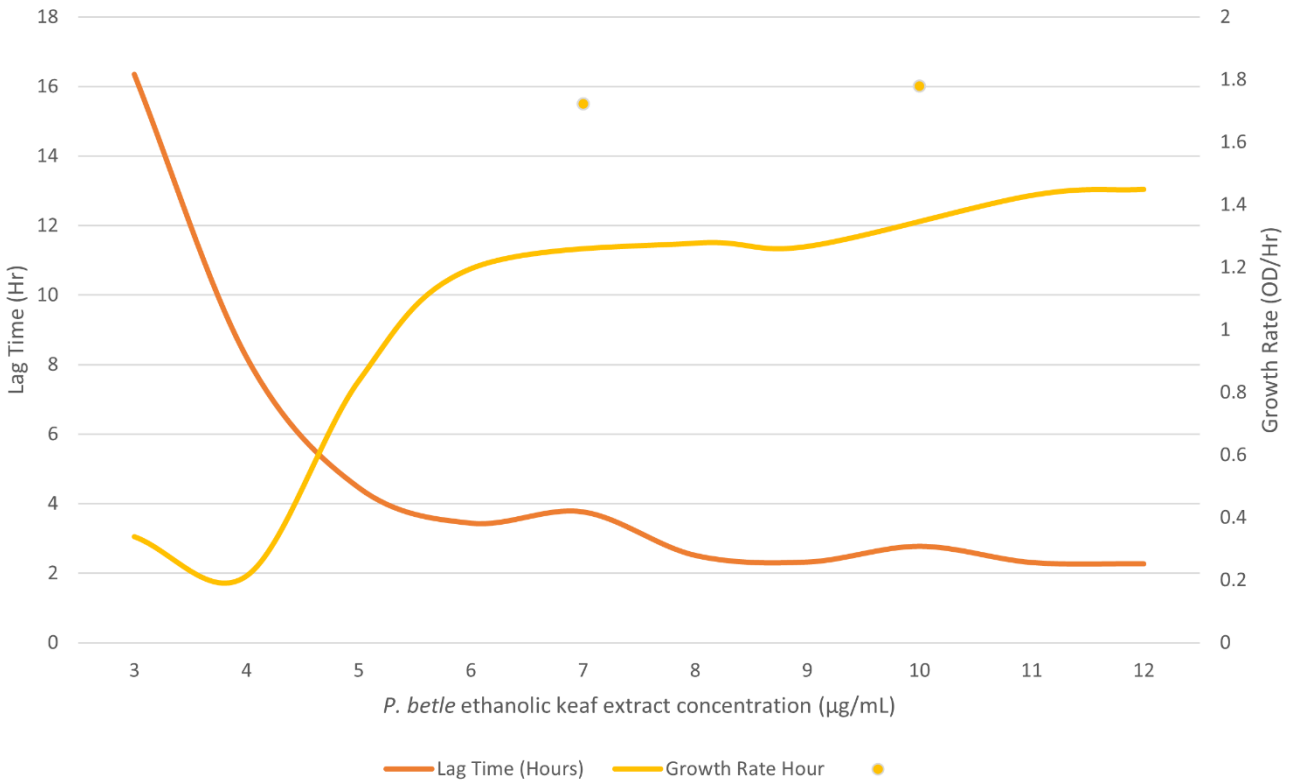


Note: Differences in the growth curves of S. aureus at subinhibitory concentrations of the extract are highlighted especially between the growth control and PBE at 1250 µg/mL.

Lag time durations increased as the extract concentration increased, with the highest effect observed at 1250 µg/mL that prolonged the lag phase by almost six (6) hours comparing against the growth control as seen in Figure 2. This is almost four (4) times longer than the growth control with no treatment added.

Figure 2

MSSA growth rates and lag time in hours in the presence of decreasing concentrations of PBE.



*Note: *p < 0.05. As the concentration of the extract rose, the durations also increased. The most significant impact was noted at 1250 µg/mL, leading to an extension of the lag phase by nearly 6 hours compared to the growth control. Outliers are left as points in the graph.*

Table 1

Mean growth parameters of surviving MSSA in different concentrations of PBE

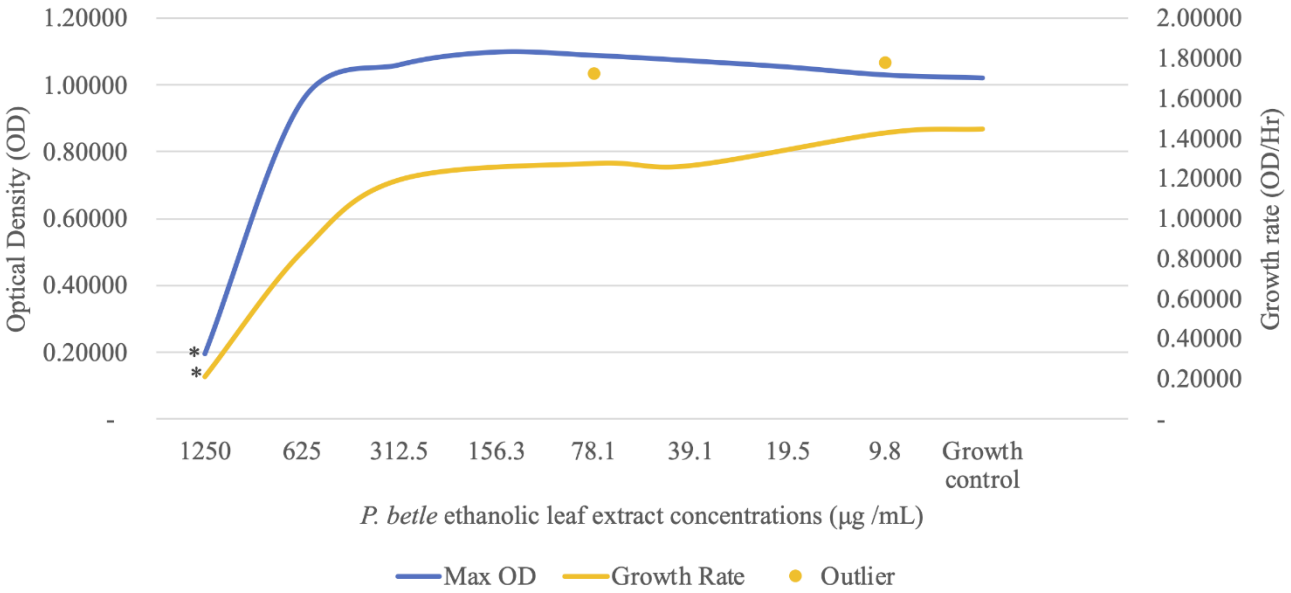
Piper betle ethanolic leaf extract (µg/mL)	Lag time (Hr)	p values vs Growth control	Growth Rate (OD/Hr)	p values vs Growth control	Max Optical Density (OD)	p values vs Growth control
1250.0	8.22*	0.001	0.214*	0.000	0.195*	0.000
625.0	4.46	0.803	0.837	0.083	0.953	0.105
312.5	3.44	0.998	1.196	0.970	1.062	0.811
156.3	3.76	0.985	1.723	0.948	1.100*	0.042
78.1	2.51	1.000	1.278	0.999	1.090	0.120
39.1	2.32	1.000	1.267	0.998	1.074	0.458
19.5	2.77	1.000	1.778	0.836	1.055	0.938
9.8	2.31	1.000	1.430	1.000	1.031	1.000
Growth control	2.27		1.450		1.022	

*Note: *p value <0.05 against growth control.*

In its effect on growth rate, all concentrations from 9.8 µg/mL up to the highest concentration of 1250 µg/mL showed a decreasing trend. Only the growth rate at 1250 µg/mL (i.e., 0.214 ± 0.021 OD/hr) was significantly different from the uninhibited growth control (i.e., 1.450 ± 0.401 OD/hr). Although at these subinhibitory concentration *S. aureus* growth is expected to persist, there is a significant decrease in the 1250 µg/mL in the maximum optical density of MSSA as compared to the growth control by as much as 80%. Although not statistically significant, there was also an observed decrease at concentration of 625 µg/mL (p=0.083). The comparison of the growth rate and the maximum optical density can be seen in Figure 3. Table 1 shows the average data computed from GrowthRates v6.2.

Figure 3

MSSA growth rates and maximum optical densities in the presence of decreasing concentrations of the PBE. Outliers are left as points in the graph



Note: Concentrations ranging from 9.8 µg/mL to 1250 µg/mL demonstrated a decreasing trend in growth rate, with only the growth rate at 1250 µg/mL significantly differing from the uninhibited control.

The MBC at 5000 µg/mL was used to describe the bactericidal effect of PBE (Lao et al., 2022). After the addition of the extract, it had exerted its bactericidal effect as early as the 2nd hour which continued to decrease until no more growth was observed after four (4) hours as seen in Table 2 and as plotted in Figure 4 and Figure 5. For the purpose of representing growth, a value of 20 colonies was used instead to compute for the CFU/mL at 4th hour. After the 4th hour, no growth or regrowth was observed, hence the CFU/mL was not computed and was assigned a value of 1. These observations were in contrast to a growth control from the previous observations wherein after lag time of approximately two (2) hours, MSSA exponentially grew until its stationary phase. Percent reduction is computed using the CFU/mL at 0h as baseline.

Table 2

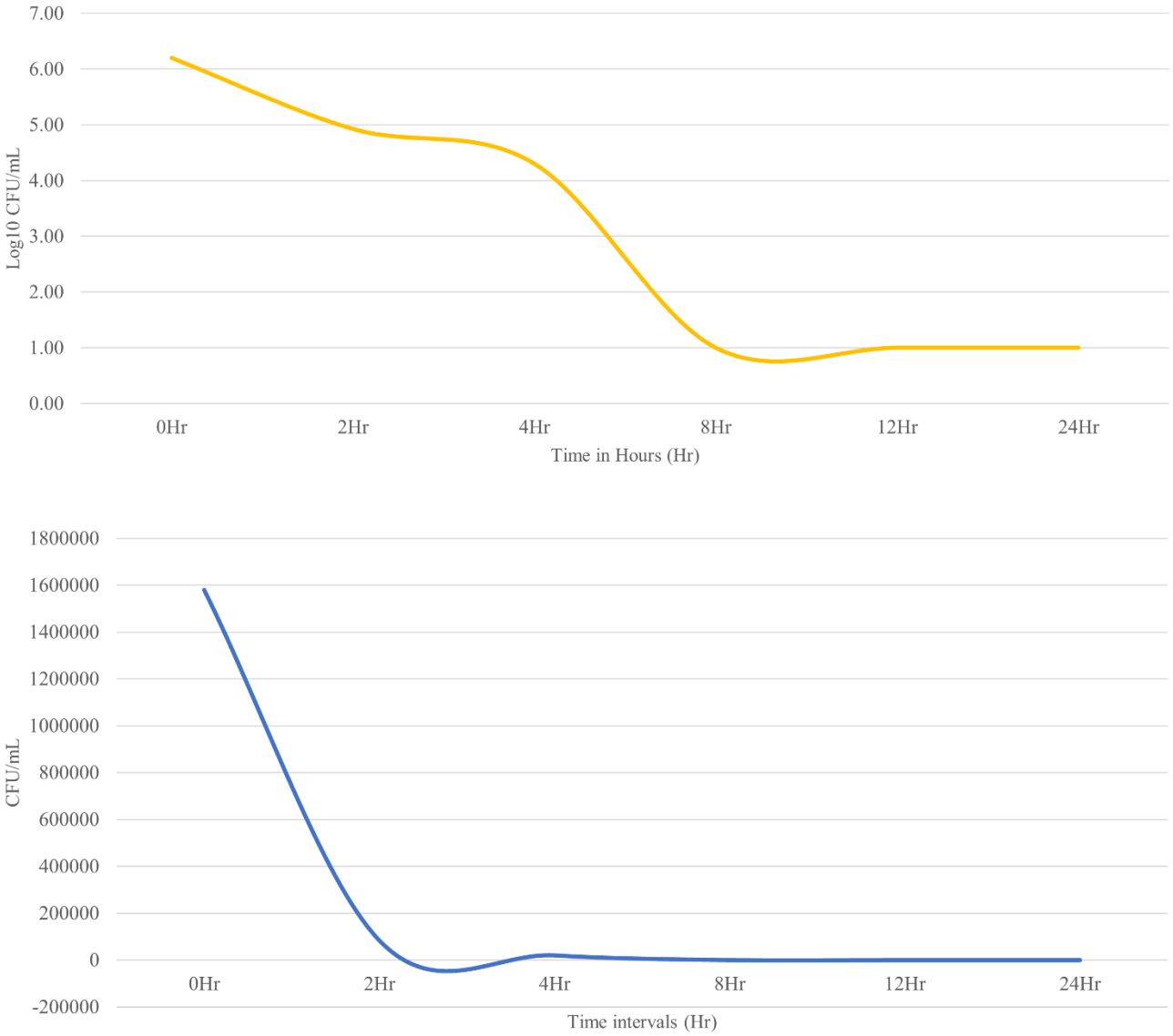
Surviving MSSA in different time intervals after being exposed to minimum bactericidal concentration of PBE

Time	CFU/mL	Log_{10} CFU/ml	% reduction
0 h	1.58×10^6	6.20	0
2 h	8.40×10^4	4.92	95%
4 h	$< 2.00 \times 10^4$	< 4.30	99%
8 h	0	1	-
12 h	0	1	-
24 h	0	1	-

Note: PBE exhibited a bactericidal effect starting from the 2nd hour, progressively declining until complete inhibition of growth after four hours. A value of 20 colonies was used to compute for the CFU/mL at 4th hour.

Figure 4

Log10 CFU/mL (A) and CFU/mL (B) of surviving MSSA in different time intervals after being exposed to MBC of PBE. Comparison of Log10 CFU/mL of MSSA with and without PBE shown in Figure 5.



Note: The bactericidal effect of PBE at the MBC of 5000 µg/mL was evident, with its action initiating as early as the 2nd hour, steadily decreasing until no growth was observed after four hours.

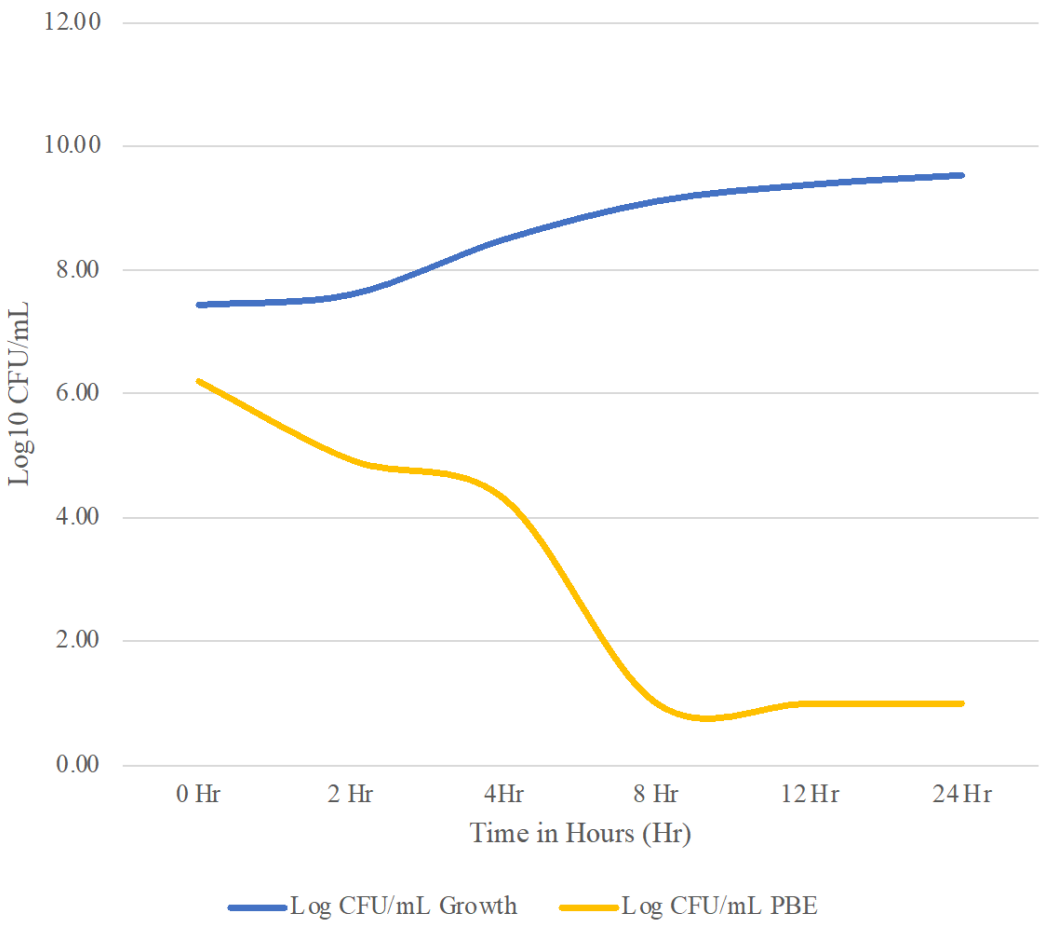
DISCUSSION

In the time-kill kinetics, results showed that even at subinhibitory concentration or 0.5 x MIC (i.e., 1250 µg/mL), PBE had significantly exerted effects on prolonging the lag time duration, slowing down the growth rate, and decreasing the maximum optical density of MSSA (Table 1, Figures 1-3). MSSA exponential growth had been delayed by six (6) hours while the growth rate was almost seven (7) times slower (i.e., 6.78x) as compared to growth control. The maximum optical density of MSSA when compared to the growth control was also lower by as much as 80% (i.e., 80.9%). It is good to note that at the minimum inhibitory concentration and above, no growth was detected on the wells suggesting growth inhibition and/or killing.

Antimicrobials have been shown to produce effects even at doses below minimum inhibitory concentrations, with varying effects to bacterial virulence as well (Braga et al., 1999; Odenholt, 2001; Otto et al., 2013; Zhanel et al., 1992). Five main aspects of bacterial virulence can be changed in MSSA after exposure to subinhibitory concentrations of antibiotics and may explain the observed effects on different parameters in the current study. These aspects are altered or deformed bacterial cells that stimulate host immune responses, unusual virulence factors that alter disease progression, modified bacterial adhesion and invasion that affect colonization and diffusion, varied biofilm formation that can potentiate or diminish device-associated infections, and increased small colony variant formation that causes persistent or recurrent infections (Chen et al., 2021).

Figure 5

Comparison of Log10 CFU/mL of MSSA with and without PBE.



Note: The uninhibited growth curve in the graph was derived from the data in a growth inhibitory assay and serves only as a visual comparator.

The implications of these findings are significant, as they underscore the relevance of subinhibitory concentrations in maintaining the desired therapeutic effects of antimicrobials. In clinical settings, dosing intervals are often extended, potentially leading to subinhibitory concentrations in vivo, especially in tissue infections where antimicrobial concentrations may be lower than in the bloodstream (Braga et al., 1999). This concept aligns with the notion of subinhibitory postantibiotic effect (PA SME), which describes the sustained inhibitory effect on bacterial growth even at doses lower than bactericidal concentrations (Odenholt, 2001). In addition to delaying bacterial regrowth, PA SME may render the remaining bacteria more susceptible to innate immunity and diminish their virulence (Eagle & Musselman, 1949). This understanding of PA SME could guide the optimization of antimicrobial dosing regimens, ensuring that intervals between doses include concentrations with bactericidal effects and durations in which an impact on bacterial growth is observed, even at subinhibitory concentrations (Eagle et al., 1950). Although the results may be correlated to PA SME, the assay specific to measuring this is outside of the current study's scope (Odenholt, 2001).

The time-kill kinetics was also done using the minimum bactericidal concentration at 5000 µg/mL to further describe the killing rate. At these concentrations, PBE has shown immediate effect on the bacterial growth from 2nd hour and continued to decrease until no further growth appeared from 8th hour to 24th hour (Table 2). This is in contrast to the growth control wherein after lag time of 2 hours, MSSA exponentially grew until it reached the stationary phase. This type of assay was done similarly by another study using 0, 25, 50, and 100 µg/mL of PBE, wherein MSSA death phase started only after 6th hour of exposure to doses above the MIC (i.e., 50 and 100 µg/mL). The observed death phase was four (4) hours later than the current study (Datta et al., 2011). Contrastingly, the current study posed some differences from the former. First, the current study quantitatively measured the MBC. Secondly, the subinhibitory effects of PBE were elaborated. Lastly, the duration of the time-kill kinetics study was for 24 hours which can capture possible persistent MSSA colonies. This is different former study which only observed the growth curves for 15 hours.

Together with the MSSA growth curves exposed at subinhibitory concentrations, the pharmacodynamics displayed by PBE suggests concentration-dependent killing. This is inferred by the increased antibacterial effects of the extract as its concentration increases from the subinhibitory concentrations up to the MBC. Furthermore, presence of antibacterial effects at subinhibitory concentrations suggest PA SME that is common in most antibiotics with concentration-dependent killing. However, more *in vivo* and pharmacokinetics or pharmacodynamic studies (e.g., time above MIC, AUC to MIC ratio, post-antibiotic effect) are needed to confirm *P. betle*'s concentration-dependent killing (Jacobs, 2001). These observed effects of PBE on MSSA growth dynamics have implications for the development of new treatment strategies. By understanding the concentration-dependent effects of PBE, researchers can explore optimal dosing regimens that maximize its antimicrobial activity. Fine-tuning the concentration and exposure duration of PBE can help achieve desired therapeutic effects while minimizing the risk of resistance development and adverse effects.

As for the specific mechanism of action, this has not been fully elucidated yet for *P. betle* in literature. Hydroxychavicol and eugenol are two primary compounds that have been linked to *P. betle*'s antibacterial activity. Both of these compounds have exhibited mechanisms resulting to bactericidal effects which include ATPase inhibition, ROS generation, membrane damage, DNA damage, and apoptosis-like death induction (Das et al., 2016; Hyldgaard et al., 2012; Singh et al., 2018).

CONCLUSION

Based on the time-kill kinetics, *PBE* has a bactericidal effect that started immediately in two (2) hours which was sustained until no growth was observed from the eighth hour and beyond. Subinhibitory concentrations have also elucidated its concentration-dependent antibacterial activity by maintaining a trend of increasing lag time, decreasing growth rate, and decreasing maximum optical density as the concentration increases with consistent significant findings particularly at 1250 µg/mL or 0.5 x MIC. Although more data and assays are required to fully correlate the observations to the subinhibitory post-antibiotic effect, the time-kill kinetics described in the current study may serve as a guide in designing in vivo antibacterial assays and provide insights into subsequent observed antibacterial effects. Isolation of the bioactives from the extract and conducting in vivo assays to further elucidate the antibacterial activities of PBE against MSSA and MRSA are highly recommended.

AUTHOR CONTRIBUTIONS

Ryan Christopher Co Lao was responsible on the conceptualization of work, acquisition and analysis of data, drafting, and revising. Dr. Ailyn Manglicmot Yabes was involved in conceptualization of the work, drafting, revising, and final approval of the version to be published.

ETHICS APPROVAL

This research was reviewed by the University of the Philippines (UP) Manila Review Ethics Board with code 2021-018-EX. It was also registered with the protocol code RGAO-2022-0296 and underwent review and approval process by the Institutional Biosafety and Biosecurity Committee with protocol number 2022-008.

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CONFLICTS OF INTEREST

All the authors declared no conflict of interest.

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